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Isotopically Labelled Adenosine Triphosphate Analogues: Synthesis of [6-^{15}N]- Adenylyl Imidodiphosphate and [6-^{15}N]-Adenylyl Methylenediphosphonate.

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Abstract

We report the synthesis of [6-^{15}N]-labeled-AMP following a novel route from adenosine with an overall yield (12%) better than with a previously described procedure from inosine (6%). The two analogues of [6-^{15}N]-adenosine triphosphate, adenylyl imidodiphosphate (AMP-PNP) and adenylyl methylenediphosphonate (AMP-PCP), were prepared from labeled AMP.

Key Words

Nitrogen-15 labelling, ATP analogues, [6-^{15}N]-labeled adenosine monophosphate from adenosine, [6-^{15}N]-adenylyl imidodiphosphate, [6-^{15}N]-adenylyl methylenediphosphonate.

Introduction

NMR studies that use adenosine triphosphate labeled with ^{15}N are expected to give valuable information regarding protein-nucleotide interactions. The purine N-6 atom is a good candidate because it can form hydrogen bonds with suitable acceptors of enzymes. We have recently reported the synthesis of [1-^{15}N] and [6-^{15}N]-labeled adenosine 5'-monophosphate (1), and shown that these ^{15}N-labels and their triphosphate derivatives could be employed to study the binding of ATP and AMP to *E. coli* adenylate kinase by ^{1}H and ^{15}N-NMR spectroscopy (2). We now describe a new route for a larger scale preparation of [6-^{15}N]-AMP from adenosine (Scheme 1). [6-^{15}N]-AMP 7 is

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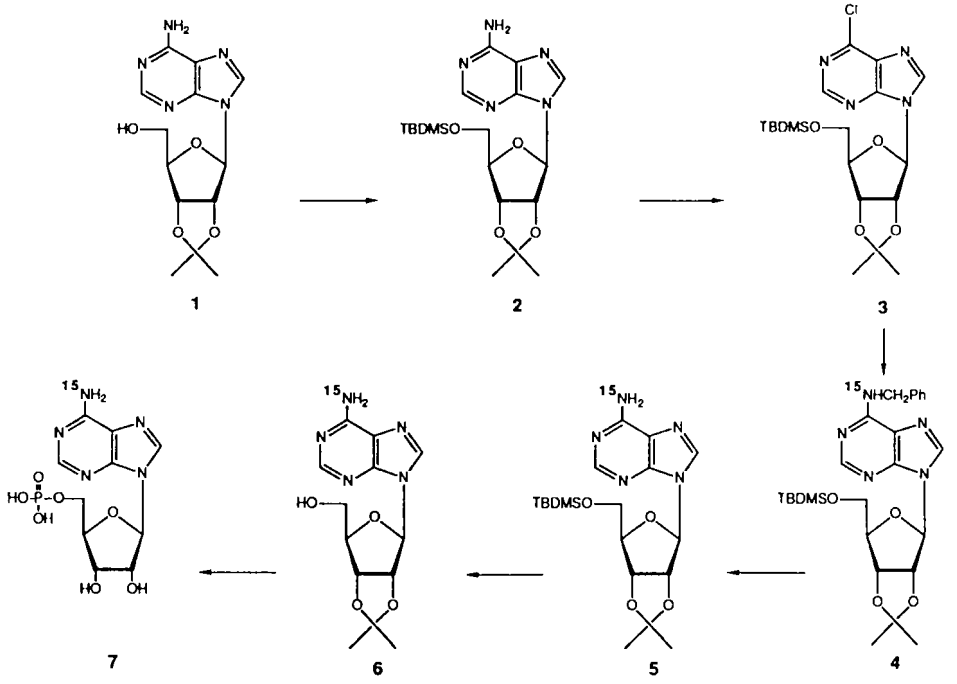
synthesized in a better overall yield via a 6-chloropurine derivative **3**. [6-^{15}N]-AMP was used as the starting material of two analogues of ATP, [6-^{15}N]-adenylyl imidodiphosphate, and [6-^{15}N]-adenylyl methylene-diphosphonate, in which the triphosphate chain is respectively less and more stable than the phosphoanhydride link. Such analogues have indeed been used as competitive inhibitors of the protein-nucleotide complex (3).

Scheme 1



Synthesis of 5'-monophosphate-[6-^{15}N]-adenosine ([6-^{15}N]-AMP).

In a previous work, we have described the synthesis of [6-^{15}N]-AMP in 6% yield from inosine (1). From this procedure, a mixture of O-sulfonated and N-sulfonated inosine in a ratio of 3:7 was obtained as intermediate. Further nucleophilic displacement of the O-sulfonyl group with ^{15}N-benzylamine gave [6-^{15}N]-AMP in a 6% overall yield. This paper describes a seven-step synthesis of [6-^{15}N]-AMP from adenosine according to Scheme 1. Thus, reaction of 2',3'-O-isopropylidene adenosine **1** with *tert*-butyldimethylsilyl chloride in the presence of imidazole in N,N-dimethylformamide gave the 5'-protected nucleoside **2** in 95% yield. Conversion of **2** into the 6-chloropurine derivative was performed via purinyl radicals (4). Thus, treatment of

compound **2** in dry carbon tetrachloride with *tert*-butyl nitrite under visible light afforded **3** in 63% yield. Nucleophilic displacement of the chloro group with ^{15}N-labeled benzylamine (5) in tetrahydrofuran with triethylamine afforded **4** in 78% yield. Debenzylation of **4** was performed using a mixture of $NaIO_4$ (4 eq) and RuO_2, x H_2O (0.02 eq) as an oxidant in a mixture of CH_2Cl_2 : CH_3CN : H_2O (2:2:3) to give directly compound **5**. We assume that ammonolysis of the benzamide formed by oxidation also occurs under these conditions. Treatment of compound **5** with a 1M solution of tetrabutylammonium fluoride in THF led to the required intermediate **6**. [6-^{15}N]-AMP **7** was finally obtained according to the Tener's procedure (6). Our method afforded [6-^{15}N]-AMP **7** from adenosine in an overall yield of 12% as compared to 6% from inosine (1).

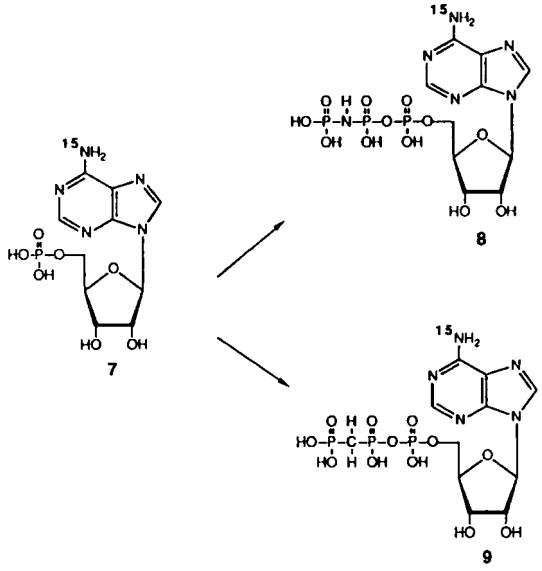
Synthesis of 5'-imido-β-γ-triphosphate-[6-^{15}N]-adenosine ([6-^{15}N]-AMP-PNP).

The [6-^{15}N]-AMP-PNP **8** was obtained according to the Michelson's procedure (7) (Scheme 2). Thus, reaction of [6-^{15}N]-AMP' **7** with chlorodiphenylphosphate in DMF, followed by displacement with the tri-*n*-butylammonium salt of imidodiphosphate, gave the ^{15}N-labeled AMP-PNP **8**. The triphosphate analogue **8** was purified by chromatography on a DEAE-cellulose column (HCO_3^- form) eluted with a 0-0.4M linear gradient of triethylammonium bicarbonate buffer (pH 7.5), followed by HPLC on a DEAE-5-PW column eluted with a 0.1M LiCl solution. The purified triphosphate was isolated in 28% yield and stored as its tetralithium salt at -20°C.

Synthesis of 5'-methylene-β-γ-triphosphate-[6-^{15}N]-adenosine ([6-^{15}N]-AMP-PCP).

The [6-^{15}N]-AMP-PCP **9** was obtained according to the simple procedure reported by Whitesides (8) (Scheme 2). Reaction of [6-^{15}N]-AMP **7** with 1,1'-carbonyldiimidazole in acetonitrile, followed by displacement with the mono-tri-*n*-butylammonium salt of methylenediphosphonate afforded the ^{15}N-labeled AMP-PCP derivative **9**. Purification by chromatography on a DEAE-cellulose column (HCO_3^- form) eluted with a 0-0.4M linear gradient of triethylammonium bicarbonate buffer (pH 7.5) gave compound **9**. After lyophilisation, the triphosphate was isolated by the Moffatt's procedure (9) as its tetrasodium salt (23% yield), which was found homogeneous on analytical HPLC on a DEAE-5-PW column eluted with a 0.15M LiCl solution.

Scheme 2



Experimental Part

Dimethylformamide (DMF), pyridine and triethylamine were distilled from CaH_2 under nitrogen and stored over activated molecular sieves (4Å). Toluene and tetrahydrofuran (THF) were distilled from Na and stored over activated molecular sieves (4Å). Carbon tetrachloride was freshly distilled. ^{15}N-Benzylamide (99.3 atom % ^{15}N, MSD Isotopes, Merck) was a generous gift from Dr D. Cowburn. ^{15}N-Benzylamine was prepared by borane reduction of ^{15}N-benzylamide (5). The ^{1}H-NMR spectra were recorded on a Bruker AC-300 instrument at 300 MHz. Chemical shifts were reported (δ ppm) relative to DMSO used as internal standard. The ^{31}P-NMR spectra were recorded on a Bruker AC-300 instrument at 121 MHz. Chemical shifts were reported (δ ppm) relative to H_3PO_4 used as external standard. Chemical ionisation mass spectra (CI) were measured on a NERMAG R10-10C apparatus. Fast atom bombardment (FAB) were recorded on a VG 70-250 double focussing instrument. Purification and analysis by HPLC were performed on a Perkin-Elmer Series 4 instrument using a gel filtration column (Bio-Gel TSK DEAE-5-PW) with a LiCl solution as eluant. TLC was performed on Merck Kieselgel F_{254} precoated plates. All compounds had analytical data of C, H, N within ± 0.4% of the theorical values.

5'-O-*tert*-Butyldimethylsilyl-2',3'-O-isopropylidene-adenosine 2.
2',3'-O-isopropylidene adenosine 1 (11.5 g, 37.5 mmol) and imidazole (6.12 g, 90 mmol) were dried by coevaporation with anhydrous toluene, and suspended in anhydrous DMF (50 ml). *Tert*-butyldimethylsilyl chloride (12.4 g, 45 mmol) was added, and the reaction mixture was stirred overnight at room temperature. The solvent was removed by evaporation. The residue was dissolved in CH_2Cl_2 and chromatographed on SiO_2, eluting with a 0 to 2% gradient of MeOH in CH_2Cl_2 to give 2 (19.3 g, 95%).

9-(5'-O-*tert*-Butyldimethylsilyl-2',3'-O-isopropylidene)-6-chloro-β-D-ribofuranosylpurine 3.
To a stirred solution of *tert*-butyl nitrite (5.3 ml, 44.2 mmol) in freshly distilled CCl_4 (400 ml) at reflux, compound 2 (7.3 g, 19.7 mmol) was added under a nitrogen atmosphere over 20 min. Reflux was maintained for 2 h under constant illumination provided by an unfrosted 200W tungsten lamp. The solvent was then removed in vacuo, the residue dissolved in CH_2Cl_2, the insoluble material filtered off and the filtrate concentrated to dryness. The residue was chromatographed on SiO_2, eluting with a 0 to 2% gradient of MeOH in CH_2Cl_2 to give compound 3 (5.27 g, 63%). MS (CI, NH_3) m/z: 443, 442, 441 $(M+H)^+$, 407. ^{1}H-NMR (DMSO, 300 MHz): δ 0.01 (s, 6H, CH_3); 0.74 (s, 9H, tBu); 1.36 (s, 3H, C-CH_3); 1.57 (s, 3H, C-CH_3); 3.70 (dd, 1H, H-5', $J_{5',5''}$ = 5.4 Hz, $J_{5',4'}$ = 10.7 Hz); 3.81 (dd, 1H, H-5", $J_{5'',5'}$ = 5.4 Hz, $J_{5'',4'}$ = 12.5 Hz); 4.37 (m, 1H, H-4'); 4.96 (dd, 1H, H-3', $J_{3',2'}$ = 6.2 Hz, $J_{3',4'}$ = 2.5 Hz); 5.45 (dd, 1H, H-2', $J_{2',3'}$ = 6.2 Hz, $J_{2',1'}$ = 1.8 Hz); 6.31 (d, 1H, H-1', $J_{1',2'}$ = 1.8 Hz); 8.80 (s, 1H, H-8); 8.84 (s, 1H, H-2).

9-(5'-O-*tert*-Butyldimethylsilyl-2',3'-O-isopropylidene)-[6-^{15}N]-benzyladenosine 4.
To a solution of 3 (4.93 g, 11.2 mmol) in fresly distilled THF (45 ml), were added ^{15}N-benzylamine (1.4 g, 13.0 mmol) and triethylamine (1.2 ml). Reflux was maintained for 18 h. The solvent was removed, the residue dissolved in CH_2Cl_2, washed with water, dried (Na_2SO_4) and concentrated to dryness. The residue was chromatographed on SiO_2, eluting with CH_2Cl_2 to give 4 (4.46 g, 78%). MS (CI, NH_3) m/z: 513 $(M+H)^+$. ^{1}H-NMR (DMSO, 300 MHz): δ -0.05 (s, 6H, CH_3); 0.80 (s, 9H, tBu); 1.34 (s, 3H, C-CH_3); 1.55 (s, 3H, C-CH_3); 3.68 (dd, 1H, H-5', $J_{5',5''}$ = 5.4 Hz, $J_{5',4'}$ = 10.7 Hz); 3.76 (dd, 1H, H-5", $J_{5'',5'}$ = 5.4 Hz, $J_{5'',4'}$ = 12.5 Hz); 4.24 (m, 1H, H-4'); 4.72 (s, 2H, CH_2); 4.99 (dd, 1H, H-3', $J_{3',2'}$ = 6.2 Hz, $J_{3',4'}$ = 2.5 Hz); 5.44 (dd, 1H, H-2', $J_{2',3'}$ = 6.2 Hz, $J_{2',1'}$ = 1.8 Hz); 6.21 (dd, 1H, H-1', $J_{1',2'}$ = 1.8 Hz); 7.30 (m, 5H, Ph); 8.24 (s, 1H, H-8); 8.32 (s, 1H, H-2); 8.50 (bd, 1H, ^{15}NH, J^{15}_{N-H} = 90 Hz).

5'-O-*tert*-Butyldimethylsilyl-2',3'-O-isopropylidene-[6-^{15}N]-adenosine 5.

To a biphasic solution of 4 (7.07 g, 11 mmol) in CH_2Cl_2: CH_3CN: H_2O (30:30:3, v/v/v) were added sodium periodate (2.42 g, 11.3 mmol) and a catalytic amount of RuO_2, x H_2O (15 mg). The reaction mixture was stirred at room temperature for 18 h. The solvents were removed, the residue dissolved in CH_2Cl_2, washed with water, dried over Na_2SO_4 and evaporated to dryness. The crude material was treated at 60°C with a mixture of concentrated ammonia : methanol (6N) for 2 h. Purification by chromatography on a silica gel column (CH_2Cl_2: MeOH, 98:2) gave compound 5 (4.2 g, 70%). MS (CI, NH_3) m/z: 423 $(M+H)^+$. ^{1}H-NMR (DMSO, 300 MHz): δ 0.03 (s, 6H, CH_3); 0.80 (s, 9H, tBu); 1.35 (s, 3H, C-CH_3); 155 (s, 3H, C-CH_3); 3.70 (dd, 1H, H-5', $J_{5',5''}$ = 5.4 Hz, $J_{5',4'}$ = 10.7 Hz); 3.76 (dd, 1H, H-5", $J_{5'',5'}$ = 5.4 Hz, $J_{5'',4'}$ = 12.5 Hz); 4.25 (m, 1H, H-4'); 4.98 (dd, 1H, H-3', $J_{3',2'}$ = 6.2 Hz, $J_{3',4'}$ = 2.5 Hz); 5.45 (dd, 1H, H-2', $J_{2',3'}$ = 6.2 Hz, $J_{2',1'}$ = 1.8 Hz); 6.20 (d, 1H, H-1', $J_{1',2'}$ = 1.8 Hz); 7.39 (d, 1H, ^{15}NH, J^{15}_{N-H} = 90 Hz); 8.19 (s, 1H, H-8); 8.29 (s, 1H, H-2).

2',3'-O-Isopropylidene-[6-^{15}N]-adenosine 6.

To a solution of compound 5 (4.10 g, 7.5 mmol) in fresly distilled THF (30 ml) was added a 1M solution of TBAF in THF (9.5 ml, 9.5 mmol). After 30 min at room temperature, the solvent was removed, the residue purified by chromatography on silica gel column (AcOEt: EtOH, 97:3) to give compound 6 (1.84 g, 79%) as a white cristalline solid. ^{1}H-NMR (DMSO, 300 MHz): δ 1.34 (s, 3H, C-CH_3); 1.56 (s, 3H, C-CH_3); 3.55 (m, 2H, H-5' and H-5"); 4.22 (m, 1H, H-4'); 4.97 (dd, 1H, H-3', $J_{3',4'}$ = 2.3 Hz, $J_{3',2'}$ = 5.6 Hz); 5.28 (bs, 1H, OH-5'); 5.35 (dd, 1H, H-2', $J_{2',3'}$ = 5.6 Hz, $J_{2',1'}$ = 3 Hz); 6.13 (d, 1H, H-1', $J_{1',2'}$ = 3 Hz); 7.39 (d, 1H, ^{15}NH, J^{15}_{N-H} = 90 Hz); 8.17 (s, 1H, H-8); 8.36 (s, 1H, H-2).

5'-Monophosphate-[6-^{15}N]-adenosine 7.

Compound 6 (1.84 g, 5.97 mmol) was converted into the corresponding [6-^{15}N]-AMP 7 according to the procedure described by Tener (6). Purification on a column of Dowex 1X2 (formate form) eluted with 0.1M formic acid gave 7 (0.94 g, 45%). The spectral characteristics are identical to those previously described (1).

5'-Imido-β-γ-triphosphate-[6-^{15}N]-adenosine 8.

[6-^{15}N]-AMP (0.35 g, 1 mmol) was converted into [6-^{15}N]-AMP-PNP according to the procedure described by Yount (3). After purification on a DEAE-cellulose (HCO_3^-) column eluted with a 0-0.4M linear gradient of triethylammonium bicarbonate buffer (pH 7.5) at 2°C [general procedure of Moffatt (9)], the tubes containing the AMP-PNP peak were collected and evaporated to dryness. The residue was further purified by HPLC on a gel

filtration column (Bio-Gel DEAE-5-PW) using 0.1M LiCl as eluant at a flow rate of 1 ml/min. The fractions containing AMP-PNP were pooled and lyophilised, the residue was washed with ethanol until the supernatants gave a negative chlorine test. [6-^{15}N]-AMP-PNP was strored frozen at -20°C as its lithium salt (0.15 g, 28%). Hydrolysis of [6-^{15}N]-AMP-PNP into adenylyl phosphoramidate in D_2O was less than 18% after 4 days at 20°C as estimated by ^{31}P-NMR spectroscopy. MS (FAB$^+$) m/z: 532 [M (-4H$^+$ + 4Li$^+$) + H]$^+$; 526 [M (-3H$^+$ + 3Li$^+$) + H]$^+$; 520 [M (-2H$^+$ + 2Li$^+$) + H]$^+$; ^{1}H-NMR (D_2O, 300 MHz): δ 4.23 (m, 2H, H-5' and H-5"); 4.38 (m, 1H, H-4'); 4.58 (dd, 1H, H-3', $J_{3',2'}$ = 5.4 Hz, $J_{3',4'}$ = 3.9 Hz); 4.76 (t, 1H, H-2', $J_{2',1'}$ = 5.7 Hz); 6.12 (d, 1H, H-1', $J_{1',2'}$ = 5.7 Hz); 8.22 (s, 1H, H-8); 8.51 (s, 1H, H-2); ^{31}P-NMR (D_2O, 121 MHz): δ -9.67 (d, 1P, Pα, $J_{\alpha,\beta}$ = 19.7 Hz); -6.50 (dd, 1P, Pβ, $J_{\beta,\alpha}$ = 19.7 Hz, $J_{\beta,\gamma}$ = 4.5 Hz); +0.25 (d, 1P, Pγ, $J_{\gamma,\beta}$ = 4.5 Hz).

5'-Methylene-β-γ-triphosphate-[6-^{15}N]-adenosine 2.

[6-^{15}N]-AMP (0.35 g, 1 mmol) was converted into [6-^{15}N]-AMP-PCP according to the procedure reported by Whitesides (8). To the free acid of AMP suspended in a mixture of MeOH (10 ml), EtOH (10 ml), was added tri-*n*-butylamine (0.185 g, 1 mmol). The reaction mixture was heated at reflux until the solid dissolved. After evaporation, the residue was dried by coevaporation with dioxane (10 ml). To a suspension of anhydrous methylene diphosphonic acid (0.88 g, 5 mmol) in acetonitrile (3 ml) at 4°C, was added tri-*n*-butylamine (0.92 g, 5 mmol). Addition of acetonitrile to a final volume of 5 ml provided a 1M standard solution of tri-*n*-butylammonium methylene diphosphonate. The [6-^{15}N]-AMP as its tri-*n*-butylammonium salt (1 mmol) and carbonyldiimidazole (4 mmol) in acetonitrile (20 ml) was stirred for 1 day. MeOH (3 mmol) was then added. After 30 min, 4 ml (4 mmol) of the standard solution was added. After 1 day, the solvent was removed by evaporation at reduced pressure and the residue was treated with MeOH (20 ml). The resulting precipitate was separated by centrifugation and then washed with MeOH (10 ml). The combined solutions were concentrated to 20 ml and a saturated solution of NaI in acetone (15 ml) was added, followed by addition of diethylether (5 ml). The resulting precipitate containing the sodium salt of [6-^{15}N]-AMP-PCP and methylene diphosphonic acid was chromatographed on an anion-exchange column (DEAE-cellulose HCO_3^-), eluted with a 0-0.4M linear gradient of triethylammonium bicarbonate buffer (pH 7.5). The fractions containing the triphosphate were pooled, concentrated at reduced pressure to 20 ml, and a saturated solution of NaI in acetone was added (15 ml) followed by addition of diethylether (5 ml). The purity of [6-^{15}N]-AMP-PCP was checked by HPLC on a gel filtration column (Bio-Gel TSK DEAE-5-PW) with a 0.15M LiCl as eluant at a flow rate of 1 ml/min. [6-^{15}N]-AMP-PCP was

strored frozen at -20°C as its sodium salt (0.23g, 38%). MS (FAB$^+$) m/z: 595 [M (-4H$^+$ + 4Na$^+$) + H]$^+$; 573 [M (-3H$^+$ + 3Na$^+$) + H]$^+$; 551 [M (-2H$^+$ + 2Na$^+$) + H]$^+$; 529 [M (-H$^+$ + Na$^+$) + H]$^+$; 507 (M + H)$^+$; ^{1}H-NMR (D_2O, 300 MHz): δ 2.29 (t, 2H, CH_2, J ^{15}N-CH = 20 Hz); 4.18 (m, 2H, H-5' and H-5"); 4.34 (m, 1H, H-4'); 4.49 (dd, 1H, H-3', $J_{3',2'}$ = 5.4 Hz, $J_{3',4'}$ = 3.8 Hz); 4.69 (t, 1H, H-2', $J_{2',1'}$ = 5.4 Hz); 6.04 (d, 1H, H-1', $J_{1',2'}$ = 5.4 Hz); 8.15 (s, 1H, H-8); 8.45 (s, 1H, H-2); ^{31}P-NMR (D_2O, 121 MHz): δ -10.46 (d, 1P, P_α, $J_{\alpha,\beta}$ = 19.1 Hz); -9.22 (m, 1P, P_β); +15.02 (m, 1P, P_γ).

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